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APPLICATION FOR LETTERS PATENT

for

BRASSICA PYRUVATE DEHYDROGENASE KINASE GENE

Inventors:

Elizabeth-France Marillia
Jitao Zou
David C. Taylor

Attorneys:
Andrew F. Nilles
Registration No. 47,825
Allen C. Turner
Registration No. 33,041
TRASKBRITT, PC
P.O. Box 2550
Salt Lake City, Utah 84110
(801) 532-1922

TITLE OF THE INVENTION

BRASSICA PYRUVATE DEHYDROGENASE KINASE GENE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of co-pending U.S. Patent Application 10/222,075, filed August 16, 2002, the contents of which are incorporated herein in its entirety.

TECHNICAL FIELD

[0002] This invention relates to plant genes useful for the genetic manipulation of plant characteristics. More specifically, the invention relates to the identification, isolation, and introduction of genes of *Brassica* PDHK sequences.

BACKGROUND

[0003] As described in FIG. 1 of PCT International Patent Application PCT/CA98/00096 to Zou and Taylor, (PCT International Publication WO 98\35044 published August 13, 1998, the contents of the entirety of which and the corresponding U.S. patent application serial no. 09/355,912, filed October 15, 1999, are incorporated by this reference), acetyl-CoA plays a central role in mitochondrial respiration and plastidial fatty acid biosynthesis. The pyruvate dehydrogenase complex (PDC) oxidatively decarboxylates pyruvate to yield acetyl-CoA.

[0004] Plants have both mitochondrial and plastidial isoforms of the PDC (see also United States Patent 6,265,636, to Randall et al (July 24, 2001); which is also incorporated in its entirety by this reference). The mitochondrial pyruvate dehydrogenase complex plays a key role in the regulation of acetyl-CoA generation and availability of acetyl moieties for various catabolic and anabolic reactions in plant cells. The mitochondrial PDC is negatively regulated by phosphorylation of the E1α subunit by pyruvate dehydrogenase kinase (PDHK), and positively regulated by dephosphorylation of the PDC by pyruvate dehydrogenase phosphatase (PDCP). Mitochondrially-generated acetyl moieties can find their way into the respiratory tricarboxylic acid (TCA; Krebs) cycle, but also into the plastid compartment where ultimately, acetate units are used by the enzymes of the fatty acid synthesis (FAS) pathway to synthesize fatty acids. These are eventually incorporated into membrane and also storage glycerolipids.

[0005] Zou and Taylor also disclose the identification, isolation and characterization of the pyruvate dehydrogenase kinase (PDHK) (gene and cDNA) sequence from the model plant system *Arabidopsis thaliana* and the utilization of this sequence in the genetic manipulation of plants. Also disclosed is a vector containing the full-length PDHK sequence or a significant portion of the PDHK sequence from *Arabidopsis*, in an anti-sense orientation under control of either a constitutive or a seed-specific promoter, for re-introducing into *Arabidopsis* or for introducing into other plants. Zou and Taylor also provided a method to construct a vector containing the full-length PDHK sequence or a significant portion of the PDHK sequence from *Arabidopsis*, in a sense orientation under control of either a constitutive or a seed-specific promoter, for re-introducing into *Arabidopsis* or for introducing into other plants. Also disclosed were methods for modifying *Arabidopsis* and other plants to change their seed oil content, average seed weight or size, respiration rate during development, vegetative growth characteristics, flowering time or patterns of generative growth, and the period required to reach seed maturity.

[0006] As disclosed in, for example, Zou and Taylor, respiration, which involves the consumption of O_2 and the catabolism of sugar or other substrates to produce CO_2, plays a central role in the process of plant growth in providing reducing equivalents, a source of energy and an array of intermediates (carbon skeletons) as the building blocks for many essential biosynthetic processes. The intermediate products of respiration are necessary for growth in meristematic tissues, maintenance of existing phytomass, uptake of nutrients, and intra- and inter-cellular transport of organic and inorganic materials. Respiration is important to both anabolic and catabolic phases of metabolism.

[0007] The pyruvate dehydrogenase complex (PDC) is a particularly important site for regulation of plant respiration. Modification of PDC activity through manipulation of PDHK expression can result in a change in the production or availability of mitochondrially-generated acetyl-CoA or a change in the respiration rate. These changes may in turn affect seed oil content, average seed weight or size, respiration rate during development, vegetative growth characteristics, flowering time or patterns of generative growth, and the period required to reach seed maturity.

[0008] Many examples exist of successful modifications to plant metabolism that have been achieved by genetic engineering to transfer new genes or to alter the expression of existing genes, in plants. It is now routinely possible to introduce genes into many plant species of

agronomic significance to improve crop performance (*e.g.*, seed oil or tuber starch content/composition; meal improvement; herbicide, disease or insect resistance; heavy metal tolerance; etc.) (Somerville, 1993; Kishore and Somerville, 1993; MacKenzie and Jain, 1997).

[0009] The *Brassica* genus includes *Arabidopsis thaliana*. The *Brassicaceae* family is comprised of a large and diverse group of plant species which are economically very important throughout the world. Three diploid *Brassica* species (*B. rapa, B. oleracea* and *B. nigra*) have hybridized in different combinations to give rise to the three amphidiploid species (*B. napus, B. juncea,* and *B. carinata*). Other Brassica species include *B. oleifera, B. balearica, B. cretica, B. elongate, B. tourneforii,* and *B. biennis*. *B. napus* and *B. rapa* have been improved through breeding programs and are now cultivated as canola crops.

[0010] It would be an improvement in the art to isolate and sequence the PDHK gene from various useful species of plants of the *Brassicaceae*.

SUMMARY OF THE INVENTION

[0011] The invention involves the isolation, and characterization of PDHK (gene and cDNA) sequences from *Brassica* species and the utilization of these sequences in the genetic manipulation of plants.

[0012] The invention also discloses a vector containing the full-length PDHK sequence or a significant portion of PDHK sequences from the *Brassicaceae*, in an anti-sense orientation under control of either a constitutive or a seed-specific promoter, for re-introduction into *Brassica* species or for introduction into other plants.

[0013] The invention further describes a method to construct a vector containing the full-length PDHK sequence or a significant portion of the PDHK sequence from *Brassica* species, in a sense orientation under control of either a constitutive or a seed-specific promoter, for re-introducing into *Brassica* or for introduction into other plants.

[0014] The invention also provides methods of modifying *Brassica* and other plants to change their seed oil content, average seed weight or size, respiration rate during development, vegetative growth characteristics, flowering time or patterns of generative growth, and the period required to reach seed maturity.

[0015] According to one aspect of the present invention, there is disclosed isolated and purified deoxyribonucleic acid (DNA) of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and/or SEQ ID NO:4. In this aspect, SEQ ID NO:1 is the nucleotide sequence and the corresponding amino acid sequence (SEQ ID NO:5) of the *Brassica napus* PDHK cDNA. SEQ ID NO:2 is the nucleotide sequence and its corresponding amino acid sequence (SEQ ID NO:6) of the *Brassica rapa* PDHK cDNA. SEQ ID NO:3 is the nucleotide sequence and the corresponding amino acid sequence (SEQ ID NO:7) of the *Brassica oleracea* PDHK cDNA. SEQ ID NO:4 is the nucleotide sequence and the corresponding amino acid sequence (SEQ ID NO:8) of the *Brassica carinata* PDHK cDNA.

[0016] In yet another aspect of the invention, there is described a vector containing one or more of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, or a part thereof, for introduction of the gene, in an anti-sense orientation into a plant cell, and a method for preparing a vector containing one or more of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, or a part thereof, for introduction of the gene in a sense orientation, into a plant cell.

[0017] The invention also relates to transgenic plants and plant seeds having a genome containing an introduced DNA sequence of one or more of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, or a part thereof, and a method of producing such plants and plant seeds.

[0018] The invention also relates to substantially homologous DNA sequences from plants with deduced amino acid sequences of 25% or greater identity, and 50% or greater similarity, isolated and/or characterized by known methods using the sequence information of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4, as will be appreciated by persons skilled in the art, and to parts of reduced length that are still able to function as inhibitors of gene expression by use in an anti-sense or co-suppression (Jorgensen and Napoli 1994) application. It will be appreciated by persons skilled in the art that small changes in the identities of nucleotides in a specific gene sequence may result in reduced or enhanced effectiveness of the genes and that, in some applications (*e.g.*, anti-sense or co-suppression), partial sequences often work as effectively as full length versions. The ways in which the gene sequence can be varied or shortened are well known to persons skilled in the art, as are ways of testing the effectiveness of the altered genes. All such variations of the genes are therefore claimed as part of the present invention.

[0019] Stated more generally, the present invention relates to the isolation, purification and characterization of a mitochondrial pyruvate dehydrogenase kinase (PDHK) genes from the *Brassicaceae* (specifically *Brassica napus, B. rapa, B. oleracea, and B. carinata*) and identifies its utility in regulating fatty acid synthesis, seed oil content, seed size/weight, flowering time, vegetative growth, respiration rate and generation time.

[0020] The PDHK of the invention is useful in manipulating PDC activity and the respiration rate in plants. For example, as disclosed in Zou and Taylor, transforming plants with a construct containing the partial PDHK gene in an anti-sense orientation controlled by a constitutive promoter can result in increased mitochondrial PDC activity, an increased production or availability of mitochondrially-generated acetyl-CoA, and hence, an increased respiration rate.

[0021] Additionally, over-expressing the full-length PDHK in a sense orientation may reduce the activity of mitochondrial PDC, resulting in decreased respiratory rates in tissues, such as leaves or tubers, to decrease maintenance respiration and thereby increase the accumulation of biomass.

[0022] Some of the manipulations and deliverables which are possible using the PDHK gene or a part thereof, include, but are not limited to, the following: seeds with increased or decreased fatty acid and oil content; plants exhibiting early or delayed flowering times (measured in terms of days after planting or sowing seed); plants with increased or decreased vegetative growth (biomass); plants with root systems better able to withstand low soil temperatures or frost; plants with tissues exhibiting higher or lower rates of respiration; plants exhibiting an enhanced capacity to accumulate storage compounds in other storage organs (*e.g.*, tubers); plants exhibiting an enhanced capacity to accumulate biopolymers which rely on acetyl moieties as precursors, such a polyhydroxyalkanoic acids or polyhydroxybutyric acids (Padgette et al., 1997).

[0023] In another exemplary embodiment, the invention discloses a genetically transformed plant having a means for modulating mitochondrially generated acetyl-CoA and/or respiration rate in the genetically transformed plant, wherein the means is operatively linked to a promoter.

[0024] In a further exemplary embodiment, the invention involves a process for modulating mitochondrially generated acetyl-CoA and/or respiration rate in a transgenic plant. The

process includes cloning a gene encoding a *Brassica* pyruvate dehydrogenase kinase protein into a vector, positioning the gene in an anti-sense orientation, and transforming a plant with the vector.

[0025] An additional process for modulating mitochondrially generated acetyl-CoA and/or respiration rate is disclosed in an additional exemplary embodiment. The process includes cloning a gene encoding a *Brassica* pyruvate dehydrogenase kinase protein into a vector, reducing production of the *Brassica* pyruvate dehydrogenase kinase protein, and transforming the vector into a plant.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] FIG. 1. Oil content (% of seed dry weight) of T_3 seed from *B. napus* cv NEX710 transgenic lines transformed with the *B. napus* Phaseolin:Antisense PDHK construct (R) compared to the oil content of seeds from their corresponding sibling null (S) control plants. Each value represents the average of three determinations.

[0027] FIG. 2. Fatty acid composition of T_3 seed oil from *B. napus* cv NEX710 transgenic lines transformed with the *B. napus* Phaseolin:Antisense PDHK construct (R) compared to the fatty acid composition of seed oil from their corresponding sibling null (S) control plants. % Sats = % of total saturated fatty acids (hatched bars); % Mono = % of total monounsaturated fatty acids (black bars); % Poly = % total polyunsaturated fatty acids (speckled bars). Each value represents the average of three determinations.

[0028] FIG. 3. Oil content (mg total fatty acids/100 seeds) of T_3 seed from *B. napus* cv NEX710 transgenic lines transformed with the *B. napus* Phaseolin:Antisense PDHK construct (R; hatched bars) compared to the oil content of seeds from their corresponding sibling null (S; black bars) or non-transformed (NT-Con; grey bar) control plants. Each value represents the average of three determinations.

[0029] FIG. 4. Average 100-seed weight (mg) of T_3 seed from *B. napus* cv NEX710 transgenic lines transformed with the *B. napus* Phaseolin:Antisense PDHK construct (R; hatched bars) compared to the oil content of seeds from their corresponding sibling null (S; black bars) or non-transformed (NT-Con; grey bar) control plants. Each value represents the average of three determinations.

[0030] FIG. 5. Transgenic Nex710 UBP:a/sBnapusPDHK line 20-9 and its vector-only control on July 18th. The transgenic line (left hand side) is fully bolted and is ready to flower (floral buds are well-developed), while the control line (right hand side) bears several leaves only and did not flower until August 1st, 14 days later.

[0031] FIG. 6. Transgenic Nex710 UBP:a/sBnapusPDHK lines 20-7 and 32-2 9 (right hand side (rhs)) and vector-only controls (left hand side (lhs)) on August 11th. The controls are in full bloom, while the transgenic lines are well into developing siliques, and have completed the flowering stage.

BEST MODE FOR CARRYING OUT THE INVENTION

[0032] The best modes for carrying out the invention are apparent from PCT/CA98/00096 (PCT International Publication WO98\35044), incorporated herein, and from the following description of the results of tests and experiments that have been carried out by the inventors. Related technology is disclosed in the incorporated U.S. Patent 6,265,636 to Randall et al.

[0033] All plant cells undergo mitochondrial respiration and this ubiquitous process is affected by the activity of the PDC and its regulators PDHK and PDCP. As disclosed in Zou and Taylor, manipulation of PDHK activity through silencing mechanisms (e.g. anti-sense RNA technology) using plant transformation can affect, *e.g.*, PDH activity, mitochondrial respiration, seed oil content, flowering time, and growth rate.

[0034] A number of ways exist by which genes and gene constructs can be introduced into plants, and a combination of plant transformation and tissue culture techniques have been successfully integrated into effective strategies for creating transgenic crop plants. These methods, which can be used in the present invention, have been extensively reviewed elsewhere (Potrykus, 1991; Vasil, 1994; Walden and Wingender, 1995; Songstad et al., 1995), and are well known to persons skilled in the art. For example, one skilled in the art will certainly be aware that these methods include *Agrobacterium*-mediated transformation by vacuum infiltration (Bechtold et al., 1993) or wound inoculation (Katavic et al., 1994), *Agrobacterium* Ti-plasmid-mediated transformation (*e.g.*, hypocotyl (De Block et al., 1989) or cotyledonary petiole (Moloney et al, 1989) wound infection), particle bombardment/biolistic methods (Sanford et al., 1987; Nehra et al.,

1994; Becker et al., 1994) or polyethylene glycol-assisted protoplast transformation (Rhodes et al., 1988; Shimamoto et al., 1989) methods.

[0035] As will also be apparent to persons skilled in the art, and as extensively reviewed elsewhere (Meyer, 1995; Datla et al., 1997), it is possible to utilize plant promoters to direct any intended up- or down-regulation of transgene expression using constitutive promoters (*e.g.*, those based on CaMV35S), or by using promoters which can target gene expression to particular cells, tissues (*e.g.*, napin promoter for expression of transgenes in developing seed cotyledons), organs (*e.g.*, roots), to a particular developmental stage, or in response to a particular external stimulus (*e.g.*, heat shock).

[0036] Particularly preferred plants for modification according to the present invention include borage (*Borago* spp.), Canola (*B. napus*, *B. rapa*, or *B. juncea*), castor (*Ricinus communis*), cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and *Limnanthes* spp., Linola, nasturtium (*Tropaeolum* spp.), *Oenothera* spp., olive (*Olea* spp.), palm (*Elaeis* spp.), peanut (*Arachis* spp.), high erucic rapeseed (*B. napus*) germplasm, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp., wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) or other members of the *Gramineae*.

[0037] Methods of modulating PDHK content and composition in a plant is described in the incorporated U.S. Patent 6,265,636 B1 to Randall et al. (see, e.g., columns 26 through 30 and 37 through 38).

[0038] The invention is further described by use of the following exemplary embodiments.

EXAMPLE I

[0039] The PDHK gene was cloned from *Brassica napus* (cv. Quantum) (SEQ ID NO:1) by Reverse Transcription - Polymerase Chain Reaction (RT-PCR) amplification. Total RNA was extracted from young leaves (Wang and Vodkin, 1994) and cDNA produced by reverse transcription (Life Technologies, Inc., 2002, M-MLV Reverse Transcriptase page 16-25). Using this cDNA and several pairs of degenerate primers (SEQ ID NO:9 and SEQ ID NO:10) designed

from conserved segments of known PDHK amino-acid sequences from *Arabidopsis* (CAA07447) and corn (AF038585), a fragment of about 1 kb was amplified by the Polymerase Chain Reaction (PCR). The fragment was cloned into the TOPO cloning vector (pCR TOPO 2.1, Invitrogen) and fully sequenced in both orientations (DNA lab, PBI/NRC). DNA sequence analysis revealed that this amplicon shared a high degree of homology with other known mtPDHK genes.

[0040] The missing termini of the gene were subsequently amplified using a 3' and 5' Rapid Amplification cDNA Ends (RACE) kit (Life Technologies, Inc., 2002, 3' RACE system and 5' RACE system pages 21-25). The full-length gene was then produced by PCR using Vent DNA polymerase (New England Biolabs) and gene specific primers (SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15) designed from the DNA sequence information provided by the RACE-generated fragments. These primers encompassed each end of the gene, i.e., the start and stop codons. At this stage, restriction sites were also added by PCR for subsequent anti-sense insertion of the PDHK gene into expression vectors such as pSE129A bearing the napin promoter (PBI/NRC) or pBBV-PHAS with the phaseolin promoter (courtesy of DowAgro Science). Orientation of the inserted gene was verified by restriction digestions and DNA sequencing.

[0041] DNA sequence analyses showed that the *B. napus* PDHK gene has an 1104 bp long open reading frame (386 AA). It was analyzed with respect to other PDHK sequences (GenBank) available and amino-acid comparison revealed 93% and 71% identity with *Arabidopsis* and corn sequences respectively. All DNA analyses (sequence alignments, primer design, etc.) were performed using the DNASTAR Lasergene™ software package.

EXAMPLE II

[0042] The same approach employed for cDNA cloning and sequence analysis of PDHK from *Brassica napus* as described in Example I was followed for the cloning and sequence analysis of the *B. rapa* PDHK gene (SEQ ID NO:2).

EXAMPLE III

[0043] The same approach employed for cDNA cloning and sequence analysis of PDHK from *Brassica napus* as described in Example I was followed for the cloning and sequence analysis of the *B. oleracea* PDHK gene (SEQ ID NO:3).

EXAMPLE IV

[0044] The same approach employed for cDNA cloning and sequence analysis of PDHK from *Brassica napus* as described in Example I was followed for the cloning and sequence analysis of the *B. carinata* PDHK gene (SEQ ID NO:4).

EXAMPLES V-VIII

[0045] The same approach employed for cDNA cloning and sequence analysis of PDHK from *Brassica napus* as described in Example I is followed for the cloning and sequence analysis of PDHK gene from *B. nigra*, *B. juncea*, *B. oleifera*, *B. balearica*, *B. cretica*, *B. elongata*, *B. tourneforii*, and *B. biennis*.

EXAMPLE IX

[0046] The oil content of a plant (*e.g.*, borage (*Borago* spp.), Canola (*B. napus*, *B. rapa* or *B. juncea*), castor (*Ricinus communis*), cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and *Limnanthes* spp., Linola, nasturtium (*Tropaeolum* spp.), *Oenothera* spp., olive (*Olea* spp.), palm (*Elaeis* spp.), peanut (*Arachis* spp.), high erucic rapeseed (*B. napus*) germplasm, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp., wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) or other members of the *Gramineae))* is modified by first introducing an anti-sense nucleic acid construct into a plant transformation vector (*e.g.*, one including a plant promoter) to produce a suitable plant transformation vector by means known to those of skill in the art (see, e.g., columns 26 to 30 of the incorporated U.S. Patent 6,265,636 to Randall et al.) The anti-sense nucleic acid construct includes recombinant nucleic acid sequence encoding Brassica PDHK (*e.g.*, the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID

NO:3, or SEQ ID NO:4). The plant's genome is thus transformed (see, e.g., columns 33 through 37 of the incorporated U.S. Patent 6,265,636) with the modified plant transformation vector. The plant seed is grown, and oil is extracted from the resulting plant seed.

EXAMPLE X

[0047] *Brassica napus* (Bn) PDHK in an anti-sense orientation modulates seed oil content and weight in *B. napus* strain Nex 710. The full length clone of the PDHK gene (SEQ ID NO:1) was inserted in an anti-sense orientation behind a phaseolin (PHAS) promoter (*See*, U.S. Pat. 5,504,200) in BBV-PHAS vector (Dow AgroSciences) and mobilized into *E. coli* (DH 5α) and *Agrobacterium* (GV3101 pMP90).

[0048] Two PmeI restriction (NEB) sites were added to the PDHK gene (SEQ ID NO:1) for cloning. The PDHK gene (SEQ ID NO:1) was inserted in anti-sense (a/s) orientation behind the PHAS promoter to form Bn PDHK-PHAS. The full length *B. napus* (Bn) PDHK gene (SEQ ID NO:1) was obtained by RT-PCR and RACE as previously described herein. Forward primer GTTTAAACATGGCGGTGAAGAAGG (SEQ ID NO:16) and reverse primer GTTTAAACTCATGGCAAAGGCTCC (SEQ ID NO:17) were designed to add a PmeI restriction site on each end of the PDHK gene. The full length Bn PDHK gene was amplified using PCR (with Vent polymerase, NEB) with primers (SEQ ID NO:16 and SEQ ID NO:17) that added the PmeI restriction sites. The amplified PCR product (amplicon) was run on a gel and cleaned from the agarose gel using a Qiagen kit. The cleaned PCR product was cloned into a TOPO cloning vector as previously described herein, transformed into *E. coli*, grown on media containing Ampicillin and the TOPO cloning vector including the PDHK gene was obtained using DNA preps (Qiagen).

[0049] The obtained TOPO cloning vector including the PDHK gene and the pBBV-PHAS-iaaH vector were digested with PmeI available from NEB. The digested pBBV-PHAS-iaaH vector was desphosphorylated, and the digested products were cleaned by running the products on a gel and purifying. The obtained PHDK gene and digested pBBV-PHAS-iaaH were blunt ligated with T4 ligase available from NEB. The ligated vector, including the PHDK gene, was electroporated into *E. coli* strain DH 12 S cells available from BRL in a ligation mixture, and the transformed cells were grown on media containing Spectinomycin. DNA preps (Qiagen) were performed on cells containing the vector to obtain Bn PDHK-PHAS. The orientation of the PDHK

gene in the vector was checked by XhoI digestion since sense and anti-sense inserts of the PDHK gene result in different digestion patterns.

[0050] *Agrobacterium* (strain GV 3101, pMP90) was transformed by electroporation with an anti-sense (a/s) PDHK insert-containing DNA prep. The transformed *Agrobacterium* cells were grown on spectinomycin containing media, and DNA preps were collected and sequenced to check the size and orientation of the PHDK gene insert. The presence of the PHDK insert gene and its actual orientation were checked with several rounds of sequencing before plants were transformed.

[0051] *B. napus* line Nex 710 (Dow Agrosciences) was chosen as an elite line and used for transformation. About 7,000 *B. napus* explants (hypocotyls) were inoculated with the *Agrobacterium* containing the a/s PHAS: Bn PDHK construct. About 6,500 of the transformed explants formed a callus, and most of the explants formed shoots (transformed or not). After transfer of the explants in 3 different medias having gradually increasing levels of heribicide (L-PPT; 0.8-10 mg/l) for selection, 77 shoot explants were allowed to root in rooting media. Most of the explants formed roots and were PCR screened for the PDHK gene and the selection marker. 55 transformed plants were positive for PAT genes. More PCR reactions were preformed to check for the presence of the marker gene in the plant and the absence of a region of the vector that should not be inserted in the plant, *i.e.*, outside the T-DNA borders, as negative controls.

[0052] Southern analyses using the PAT gene as a probe confirmed the transformation events, gave better estimates of the success of transformation (about 0.8%), and indicated the number of copies of the transgene that were inserted. 52 plants had one or several inserts, and 42 plants were retained for having 1 or 2 copies of the transgene.

[0053] Leaf painting with 3 mg/L Liberty was performed on T_1 plantlets and 10 resistant plants per line were selected. The first generation of transgenic lines was harvested and seeds were analyzed by gas chromatography (GC) for oil content. Several lines looked promising for having up to a 10% increase of seed oil. At this stage, the populations were still genetically segregating (the transgene insertion is hemizygous) and, therefore, the data on oil content represents an average value for each population of segregating genotype. 10 plants of each of the 42 lines that produced seeds were seeded in greenhouse for production of the T_2 generation.

[0054] 42 lines were selected based on the number of inserted transgenes (1 or 2 copies of the transgene) for further generations. 10 plants per line were seeded, and the T_2 seeds were

analyzed by GC for oil content. Several promising lines were identified and exhibited increased oil content. For instance, 16 lines had a 17% increase of oil content and 1 line had a 36% increase of oil content. Other promising cell lines had increased seed weight. For instance, 38 lines had a 16% increase in seed weight and 1 line had an 80% increase in seed weight. Other lines had increased total oil content on a per seed basis. 32 lines had a 27% increase in total oil content per seed and 1 line had a 78% increase of total oil per seed as compared to the control non-transformed wild type or the respective sibling null lines.

[0055] T_3 seeds (9 transgenic lines, 10 plants each, plus vector-only, nulls and wild type control lines) were harvested and analyzed for oil content, composition and average seed weight. The T_3 data are presented in FIGS. 1-4.

EXAMPLE XI

[0056] Utility of Bn PDHK (SEQ ID NO:1) in modulating flowering (generation) time. The Bn PDHK gene (SEQ ID NO:1) was inserted in an anti-sense orientation behind the Ubiquitin (UBQ) gene promoter (*See*, U.S. Pat. 6,054,574) in the pDAB4016M vector (Dow AgroSciences). The Bn PDHK gene was obtained by amplifying the full length Bn PDHK gene (obtained by RT-PCR, reverse transcription PCR, and RACE as previously described herein) with a forward primer CGTACGATGGCGGTGAAGAAGGCTA (SEQ ID NO:18) and a reverse primer GGTGACCTCATGGCAAAGGCTCCT (SEQ ID NO:19) designed to add two restriction sites, BstE II and BsiW I, at each end of the Bn PDHK gene. The amplification was performed by PCR using Vent polymerase available from NEB. The PCR product was run on an agarose gel and cleaned using a Qiagen kit. The cleaned PCR product was cloned into a TOPO cloning vector as previously described herein and the TOPO cloning vector including the Bn PDHK gene was transformed into a cell. The transformed cell was grown on media including Ampicillin and the TOPO cloning vector including the Bn PDHK gene was obtained from the cells using DNA preps from Qiagen.

[0057] The TOPO preps and pDAB4016M vector (courtesy of Dow Agrosciences) were digested with BstE II and BsiW I restriction enzymes available from NEB. The digested pDAB4016M vector was dephosphorylated, and the digested products were cleaned by running on a gel and using a Qiagen kit. The digested pDAB4016M vector and the digested TOPO preps

including the Bn PDHK gene were blunt ligated with T4 ligase available from NEB. *E. coli* strain DH 20B cells, available from BRL, were ligated with a ligation mixture including the ligated vector. The transformed cells were grown on media including Erythromycin and DNA preps (Qiagen) were performed to obtain the a/s Bn PDHK-DAB vector.

[0058] *Agrobacterium* strain GV 3101 pMP90 was transformed by electroporation with the a/s insert-containing DNA prep. The *Agrobacterium* cells were grown on media including Erythromycin, and DNA preps were performed to obtain the a/s insert-containing vector. The a/s insert-containing vectors were sequenced to check the size and orientation of the insert. The presence of the PDHK, the PAT marker gene and the Ubiquitin promoter was verified by PCR performed on DNA obtained from transformed *Agrobacterium* DNA.

[0059] 9,000 explants (hypocotyls from the *B. napus* Nex 710 line) were transformed with *Agrobacterium* containing the a/s Bn PDHK-DAB vector (5,000 explants) or the pDAB vector as a control (4,000 explants). The transformed explants were selected on a LPPT-containing medium as known in the art, and the transformed explants were screened by PCR for the presence of the PDHK insert, the PAT gene and the UBQ promoter. 60 plants were analyzed by Southern blots (using a probe for the PAT marker gene) to check the number of transgenes that were inserted. The transformation rate was determined to be 0.9% and 37 plants with one or two copies of the insert were retained for further generations.

[0060] T_1 seeds were harvested and seeded in a greenhouse. The resulting plantlets were leaf-painted with Liberty herbicide to detect null-sib lines. 34 lines were retained (8-10 plants each) and 17 lines having a corresponding null-sib line were obtained as control, the rest of the lines had vector-only as a control.

[0061] Most of the transformed *B. napus* Nex 710 plants displayed a significant early flowering phenotype in the T_2 generation as compared to the controls. FIG. 5 shows a transgenic plant with anti-sense PDHK flowering 2 weeks earlier than the control. Thus, the transformed plant exhibits a 15% shorter cycle when assuming an average cycle of 100 days. A demonstration of early flowering by down-regulating PDHK gene in *B. napus* Nex 710, a double haploid (DH) line which is known for uniform maturity, demonstrates the utility of manipulating the expression of the PDHK gene in eliciting early flowering.

[0062] Although described with the use of particular exemplary examples and embodiments, the scope of the invention is to be determined by the appended claims.

REFERENCES:

Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C.R. Acad. Sci. Ser. III Sci. Vie*, 316: 1194-1199.

Becker, D., Brettschneider, R. and Lörz, H. (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant J.* 5: 299-307.

Datla, R., Anderson, J.W. and Selvaraj, G. (1997) Plant promoters for transgene expression. *Biotechnology Annual Review* 3: 269-296.

De Block, M., De Brouwer, D. and Tenning P. (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol.* 91: 694-701.

Jorgensen, R.A. and Napoli, C.A. (1994) Genetic engineering of novel plant phenotypes. U.S. Patent No. 5283184.

Katavic, V., Haughn, G.W., Reed, D., Martin, M. and Kunst, L. (1994) *In planta* transformation of *Arabidopsis thaliana. Mol. Gen. Genet.* 245: 363-370.

Kishore G.M. and Somerville, C.R. (1993) Genetic engineering of commercially useful biosynthetic pathways in transgenic plants. *Current Opinion in Biotechnology.* 4: 152-158.

MacKenzie, S.L. and Jain, R.K. (1997) Improvement of oils crops via biotechnology. *Recent Res. Dev. In Oil Chem.* 1: 149-158.

Meyer, P. (1995) Understanding and controlling transgene expression. *Trends in Biotechnology*, 13: 332-337.

Moloney, M.M., Walker, J.M. and Sharma, K.K. (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.* 8: 238-242.

Nehra, N.S., Chibbar, R.N., Leung, N., Caswell, K., Mallard, C., Steinhauer, L. Baga, M. and Kartha K.K. (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. *Plant J.* 5: 285-297.

Padgette, S.R., Gruys, K.J., Mitsky, T.A., Tran, M., Taylor, N.B., Slater, S.C. and Kishore, G.M. (1997) Strategies for production of polyhydroxyalkanoate polymers in plants. *Plant Physiol.* Suppl., 114: 3 (abstract 10003).

Potrykus, I. (1991) Gene transfer to plants: Assessment of published approaches and results. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42: 205-225.

Rhodes, C.A., Pierce, D.A., Mettler, I.J., Mascarenhas, D. and Detmer, J.J. (1988) Genetically transformed maize plants from protoplasts. *Science* 240: 204-207.

Sanford, J.C., Klein, T.M., Wolf, E.D. and Allen, N. (1987) Delivery of substances into cells and tissues using a particle bombardment process. *J. Part. Sci. Technol.* 5: 27-37.

Shimamoto, K., Terada, R., Izawa, T. and Fujimoto, H. (1989) Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature* 338: 274-276.

Somerville, C.R. (1993) Future prospects for genetic modification of the composition of edible oils from higher plants. *Am. J. Clin. Nutr.* 58 (2 Suppl.): 270S-275S.

Songstad, D.D., Somers, D.A. and Griesbach, R.J. (1995) Advances in alternative DNA delivery techniques. *Plant Cell, Tissue and Organ Culture* 40: 1-15.

Vasil, I.K. (1994) Molecular improvement of cereals. *Plant Mol. Biol.* 25: 925-937.

Walden, R. and Wingender, R. (1995) Gene-transfer and plant regeneration techniques. *Trends in Biotechnology* 13: 324-331.

Wang, C-S and Vodkin, L. O. (1994) Extraction of RNA from tissues containing high levels of procyanidins that bind RNA. *Plant Molecular Biology Reporter* 12: 132-145.